

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
10 May 2002 (10.05.2002)

PCT

(10) International Publication Number
WO 02/36135 A2

(51) International Patent Classification⁷: **A61K 35/00**,
45/06, 31/495

(21) International Application Number: PCT/GB01/04902

(22) International Filing Date:
6 November 2001 (06.11.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/246,233 6 November 2000 (06.11.2000) US
60/248,095 13 November 2000 (13.11.2000) US
60/345,982 19 October 2001 (19.10.2001) US

(71) Applicant (*for all designated States except US*):
PHARMA MAR, S.A. [ES/ES]; Calle de la Calera,
3, Poligono Industrial de Tres Cantos, Tres Cantos,
E-28760 Madrid (ES).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): **TAKAHASHI, Naoto** [JP/US]; Memorial Sloan Kettering 1275 York Avenue, New York, NY 10021 (US). **WEITMAN, Steve** [US/US]; Institute for Drug Development, Cancer Therapy and Research Center (CTRC), 14960 Omicron, San Antonio, TX 78245-3217 (US). **D'INCALCI, Maurizio** [IT/IT]; Institute de Rechercher Farmacologique Mario Negr, I- Milan (IT). **FAICLOTH, Glynn, Thomas** [US/US]; PharmaMar USA, Inc., 320 Putnam Avenue, Cambridge,

MA 02139-4616 (US). **GIAVAZZI, Raffaella** [IT/IT]; Institute de la Recherche Farmacologique Mario Negr, i, I-Milan (IT). **GESCHER, Andreas** [DE/GB]; 7 Swithland Court, Brand Hill, Woodhouse Eaves LE12 8SS (GB).

(74) Agent: **RUFFLES, Graham, Keith**; Marks & Clerk, 57-60 Lincoln's Inn Fields, London WC2A 3LS (GB).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: EFFECTIVE ANTITUMOUR TREATMENTS

(57) Abstract: ET-743 is used in the preparation of a medicament for an effective treatment of a tumour by combination therapy employing ET-743 with another drug.



WO 02/36135 A2

Effective Antitumour Treatments

The present invention relates to effective antitumour treatments.

Ecteinascidin 743, ET743, is an anticancer agent derived from a marine source.

BACKGROUND OF THE INVENTION

The reader is referred to WO0069441 published 23 November 2000 for information on compositions and uses of ET743 for treating cancer. This text is incorporated by reference.

SUMMARY OF THE INVENTION

In accordance with one aspect of this invention, we provide effective combination therapies based on ecteinascidin 743, using other drugs.

PREFERRED EMBODIMENTS

The other drugs may form part of the same composition, or be provided as a separate composition for administration at the same time or a different time. The identity of the other drug is not particularly limited, and suitable candidates include:

- a) drugs with antimitotic effects, especially those which target cytoskeletal elements, including microtubule modulators such as taxane drugs (such as taxol, paclitaxel, taxotere, docetaxel), podophylotoxins or vinca alkaloids (vincristine, vinblastine);
- b) antimetabolite drugs such as 5-fluorouracil, cytarabine, gemcitabine, purine analogues such as pentostatin, methotrexate);

- c) alkylating agents such as nitrogen mustards (such as cyclophosphamide or ifosfamide);
- d) drugs which target DNA such as the anthracycline drugs adriamycin, doxorubicin, pharmorubicin or epirubicin;
- e) drugs which target topoisomerases such as etoposide;
- f) hormones and hormone agonists or antagonists such as estrogens, antiestrogens (tamoxifen and related compounds) and androgens, flutamide, leuprorelin, goserelin, cyprotrone or octreotide;
- g) drugs which target signal transduction in tumour cells including antibody derivatives such as herceptin;
- h) alkylating drugs such as platinum drugs (cis-platin, carbonplatin, oxaliplatin, paraplalin) or nitrosoureas;
- i) drugs potentially affecting metastasis of tumours such as matrix metalloproteinase inhibitors;
- j) gene therapy and antisense agents;
- k) antibody therapeutics;
- l) other bioactive compounds of marine origin, notably the didemnins such as aplidine;
- m) steroid analogues, in particular dexamethasone;
- n) anti-inflammatory drugs, in particular dexamethasone; and
- o) anti-emetic drugs, in particular dexamethasone.

As part of this patent specification, we include a series of examples and now refer to them. These examples demonstrate the increased effectiveness of ET-743 when used in combination with other drugs and are concerned with different combinations using ET-743.

Example 1 relates to effective combinations of ET-743 and doxorubicin for tumour growth inhibitions against marine and human sarcomas in athymic mice.

Example 2 shows ecteinascidin 743 (ET-743) and doxorubicin produce synergistic cytotoxic effects in soft tissue sarcoma lines HT-1080 and HS-18.

These two examples show more than additive effects of the combination of ET-743 with anthracyclines (in particular doxorubicin) which is more effective than either alone against human tumours (in these specific experiments sarcoma), which effects occur independent of sequence of administration. Such results show clear promise for treatment of patients.

Example 3 shows a synergistic cytotoxic effect of ET-743 and cisplatin.

Example 4 provides a sequencing evaluation of ET-743 in combinations with chemotherapy agents against a panel of human tumour cell lines, in particular ET743 combinations with doxorubicin, taxol, SN-38, cisplatin, and gemcitabine.

These two show more than additive effects of the combination of ET-743 with platinum antitumour compounds, (in particular Cis-platin) with the nucleoside analogue gemcitabine, and with an inhibitor of topoisomerase II (SN38, which is the active agent produced from pro-drug CPT-11, a drug of the camptothecin group). Again these combinations are more effective than either drug alone against human tumours (in these specific experiments against a variety of tumour cells: ovarian, colon, lung, breast, bone sarcoma), which effects were dependent on sequence of exposure in some cases. Again there is promise for treatment of patients.

Interestingly, synergistic action was clearly not predictable: Example 4 indicates that in most combinations tested, no synergy was observed (in fact, antagonism was reported in some cases).

Example 5 relates to evaluation of combinations of Et-743 with doxorubicin or trimetrexate or paclitaxel.

It shows more than additive effects of the combination of ET-743 with anthracyclines (in particular doxorubicin) which is more effective than either alone against human tumours (in these specific experiments sarcoma), which effects occur independent of sequence of administration. Such results show clear promise for treatment of patients.

Examples 6 to 8 reinforce and complement the previous examples, and especially show the synergy of ET-743 and doxorubicin and also ET-743 with cisplatin.

Example 9 demonstrates a different kind of effectiveness of the combinations of this invention, where high-dose dexamethasone protects against the hepatotoxicity of ecteinascidin-743 (ET-743).

In summary, this invention therefore provides compositions, methods of treatment, processes for preparing compositions and related embodiments.

The present invention also extends to the compounds of the invention for use in a method of treatment, and to the use of the compounds in the preparation of a composition for treatment of cancer.

Thus, the present invention provides a method of treating any mammal, notably a human, affected by cancer which comprises administering to the affected individual a therapeutically effective amount of a compound of the invention, or a pharmaceutical composition thereof.

The present invention also relates to pharmaceutical preparations including a pharmaceutically acceptable carrier, which contain as active ingredient a compound or compounds of the invention, as well as the processes for their preparation.

Examples of pharmaceutical compositions include any solid (tablets, pills, capsules, granules, etc.) or liquid (solutions, suspensions or emulsions) with suitable composition or oral, topical or parenteral administration, and they may contain the pure compound or in combination with any carrier or other pharmacologically active compounds. These compositions may need to be sterile when administered parenterally.

Administration of the compounds or compositions of the present invention may be by any suitable method, such as intravenous infusion, oral preparations, intraperitoneal and intravenous administration. We prefer that infusion times of up to 24 hours are used, more preferably 2-12 hours, with 2-6 hours most preferred. Short infusion times which

allow treatment to be carried out without an overnight stay in hospital are especially desirable. However, infusion may be 12 to 24 hours or even longer if required. Infusion may be carried out at suitable intervals of say 2 to 4 weeks. Pharmaceutical compositions containing compounds of the invention may be delivered by liposome or nanosphere encapsulation, in sustained release formulations or by other standard delivery means.

The correct dosage of the compounds will vary according to the particular formulation, the mode of application, and the particular *situs*, host and tumour being treated. Other factors like age, body weight, sex, diet, time of administration, rate of excretion, condition of the host, drug combinations, reaction sensitivities and severity of the disease shall be taken into account. Administration can be carried out continuously or periodically within the maximum tolerated dose.

The combinations of this invention can be used on refractory patients. The reader is referred to WO0069441 for information on dosing schemes for ET-743 and other information of use in the combination therapy of this invention.

EXAMPLES OF THE INVENTION

Example 1

Effective Combinations Of Et-743 And Doxorubicin For Tumor Growth Inhibitions Against Murine And Human Sarcomas In Athymic Mice

ET-743 has confirmed clinical activity in patients with soft and bone sarcoma refractory to previous chemotherapy including Doxorubicin (Dx) and Ifosfamide. In view of the potential clinical value in combining ET-743 with Dx we have investigated this combination against the murine fibrosarcoma UV2237, its mdr-resistant subline UV2237/ADR and the human rhabdomyosarcoma xenograft TE671. Both ET743 and Dx alone were effective against murine UV2237 fibrosarcoma whereas each was inactive or marginally active against both UV2237/ADR and TE671. However, the combination of ET743 and Dx was effective in all 3 models. The synergism was particularly marked in the

human rhabdomyosarcoma TE671 and appeared independent of drug sequence or combination.

After single i.v. treatments performed when the tumor TE671 was approximately of 100 mg tumor weight inhibition (TWI) and Log 10 Cell Kill (LCK) values were respectively 46% and 0.132 for ET-743 (0.1 mg/kg) alone, 50% and 0.33 for Dx (10 mg/kg) alone, 77% and 0.924 for ET-743 (0.1 mg/kg) and Dx (10 mg/kg) given simultaneously, 82% and 1.12 for the combination of ET-743 (0.1 mg/kg) given 1 hour before Dx (10 mg/kg) and 75% and 0.85 for the combination of ET-743 (0.1 mg/kg) given 1 h after Dx (10 mg/kg).

These data suggest that the combination of ET-743 and Dx can also be effective in tumors that are not sensitive or marginally sensitive to these drugs given alone, thus providing a strong rationale for clinical investigations using this combination.

Example 2

Ecteinascidin 743 (et-743) and Doxorubicin Produce Synergistic Cytotoxic Effects in Soft Tissue Sarcoma Lines HT-1080 and HS-18.

Two sarcoma cell lines, HT 1080, a fibrosarcoma cell line sensitive to ET-743 ($IC_{50} = 10\text{pm}$) and HS-18, a liposarcoma cell line, less sensitive to ET-743 ($IC_{50} = 270\text{pm}$) were evaluated for toxicity to ET-743 in combination with either doxorubicin, trimetrexate or paclitaxel. When ET-743 was used in combination with each of these drugs at a constant molar ration, and analysed by the method of Chou and Talalay, synergistic effects were obtained (72 hr incubation) with the ET-743-doxorubicin combination, but not with the combination of ET-743 with trimetrexate or paclitaxel. When cells were exposed to ET-743 for 72 hr, and either doxorubicin, trimetrexate or taxol for the last 48 hrs of incubation, synergistic effects were also obtained with doxorubicin against both sarcoma cell lines. Of interest, the sequence paclitaxel followed by ET-743 was more effective than the opposite sequence. These results encourage clinical trials of doxorubicin in combination with ET-743 to treat patients with soft tissue sarcoma, as both of these drugs have shown activity against this disease.

Example 3

Synergistic Cytotoxic Effect Of Et-743 And Cisplatin

Ecteinascidin 743 (ET-743) has shown striking antitumor activity in several preclinical systems and promising clinical activity. ET-743 binds N2 guanines in the minor groove and affects the regulation of transcription (Minuzzo et al., PNAS, Vol. 97,6780-84, 2000).

Previous studies have indicated that mismatch repair (MMR) deficient cells are equally sensitive to ET-743 as proficient cells. NER deficient cells very sensitive to cisplatin are 6-8 times less sensitive to ET-743. On the basis of the different mechanisms involved in the repair of ET-743 and cisplatin and because of the potential clinical interest in this combination we have performed studies to evaluate the cytotoxic effects of ET-743 and cisplatin in several human tumor cell lines. Human ovarian cancer Igrove-1 cell line, a subline resistant to ET-743 (IG/PSC/ET), human colon cancer HCT 116, (MMR deficient) and HCT11-ch3 (MMR proficient) cell lines were used in this study.

The cells were treated for 1 or 24 h with different concentrations of ET-743 or cisDDP, alone or in combinations, and the cytotoxicity was evaluated by using a colorimetric assay after sulforodhamine B staining. In all the cell lines a synergistic effect was observed both with 1 h or 24 h exposure. Interestingly in HCT116 resistant to cisDDP ET-743 was apparently able to reverse sensitivity even at concentrations of ET-743 which alone were marginally effective. Taken together the data provide a rational for undertaking clinical studies combining ET-743 with cisDDP.

Example 4

Et743 Combinations With Doxorubicin, Taxol, Sn-38, Cisplatin, And Gemcitabine

ET-743 was evaluated in combination with doxorubicin, taxol, SN-38, cisplatin, and gemcitabine against a panel of human tumor cell lines. These studies were designed to determine the type of drug-drug interaction between ET-743 and standard chemotherapy agents and the influence of sequence of exposure on antitumor activity. Multiple combinations of ET-743 with standard cytotoxic agents were used with a model-free design (Laska, *et al. Biometrics* 50:834, 1994) to describe the type of drug-drug interaction. These studies suggest that regardless of exposure, an additive pattern of drug-drug interaction is most typically observed.

A synergistic drug-drug interaction was observed when ET-743 was combined against non-small cell lung (pre-exposure to SN-38), osteosarcoma (pre-exposure with ET-743 followed by cisplatin), breast (pre-exposure to ET-743 followed by gemcitabine), colon (pre-exposure with ET-743 followed by SN-38 and concurrent exposure with SN-38) tumor cell lines. An additive/synergistic (pre-exposure to ET-743 followed by SN-38 against NSCL; pre-exposure to SN-38 against colon and NSCL; concurrent exposure with cisplatin against osteosarcoma, and with SN-38 against NSCL lines) pattern of drug-drug interaction was observed. Evidence of antagonism was noted when taxol was utilized concurrently against two NSCL lines, and doxorubicin against a rhabdomyosarcoma cell line.

These studies suggest that ET-743 which is in Phase II clinical trials, could be combined with several cytotoxic agents against a broad-range of tumor types.

Material And Methods

Cell culture:

Human breast (MDA-435, MDA-231, T-470), non-small cell lung (NCI-H522, NC1-H226, NCI-H23), colon (HCT-116, HT-29, Colo-320), osteosarcoma (HOS, U-2, OS, SaOS-2), rhabdomyosarcoma (RH1, RH30, RD) tumor cell lines were grown in RPMI-1640 supplemented with 10% fetal bovine serum and 2mM L-glutamine. All stock cultures were maintained in 75 cm² flasks at 37°C in humidified incubators with a 5% CO₂-95% air atmosphere.

IC₅₀ Analysis:

A pre-determined number of exponentially growing tumor cells were inoculated in 96-well tissue culture plates and allowed to stabilize for 24 hours. Afterwards, a drug plate consisting of serial diluted concentrations of ET-743 or standard chemotherapy agents was added to the cells. Cells were incubated as a 24-hour exposure for three days followed by the addition of MTT for 4 hours. Resultant formazan crystals were then solubilized with acid/alcohol, with absorbance (570 nm-test/630 nm-reference) determined using a microplate reader. Results were expressed as percent tumor cell kill compared to media controls.

Combination Studies:

For the combination studies, the concentration (expressed as a percent of the individual agent's IC₅₀) schema used to characterize the type of interaction is shown below:

Drug Concentration (Expressed as a percent of the IC ₅₀)	
<u>ET-743</u>	<u>Standard agents</u>
100	0
75	25
60	40
50	50
40	60
25	75
0	100
0	0

Statistical Analysis of Combination Studies:

Statistical comparisons are made with each test combination (75:25-ET-743/standard agents) and the endpoints (100:0-ET-743 and 0:100-standard agents). A statistically significant observation requires that a difference exists between the combination (ET-743 and standard agents) absorbance value and both endpoint values (ET-743 and standard

agents alone). If the majority of (≥ 3 of 5) the values are statistically above or below the line then antagonism or synergy is described, respectively. Otherwise the pattern is more consistent with an additive interaction. Interpretation is very difficult if there is considerable slope to the line connecting the endpoints. If the slope of the IC₅₀ curves for the individual agents are identical (unlikely) then you can, at times, determine the type of interaction.

Sequencing Combination of ET-743 with Chemotherapy Agents		
<u>Tumor Type/Cell Line</u>	Exposure Conditions/Agents	<u>Drug-Drug Interactions Observed</u>
<u>Osteosarcoma</u>		
NOS	24 hour ET-743 followed by 24 hour exposure to cisplatin	Synergistic
	24 hour cisplatin followed by 24 hour exposure to ET-743	Additive
	24 hour concurrent ET-743/cisplatin exposure	Additive
U2-OS	24 hour ET-743 followed by 24 hour exposure to cisplatin	Additive
	24 hour cisplatin followed by 24 hour exposure to ET-743	Additive
	24 hour concurrent ET-743/cisplatin exposure	Additive
Sa06	24 hour ET-743 followed by 24 hour exposure to cisplatin	Additive
	24 hour cisplatin followed by 24 hour exposure to ET-743	Additive
	24 hour concurrent ET-743/cisplatin exposure	Additive/Synergistic
<u>Non-Small Cell Lung</u>		
	24 hour ET-743 followed by 24 hour exposure to taxol	Additive

NCB-H226	24 hour taxol followed by 24 hour exposure to ET-734	Additive
	24 hour concurrent ET-743/taxol exposure	Antagonistic
	24 hour ET-743 followed by 24 hour exposure to SN38	Additive/Synergistic
	24 hour SN-38 followed by 24 hour exposure to ET-743	Additive/Synergistic
	24 hour concurrent ET-743/SN-38 exposure	Additive
NCB-N522	24 hour ET-743 followed by 24 hour exposure to taxol	Additive
	24 hour taxol followed by 24 hour exposure to ET-734	Additive
	24 hour concurrent ET-743/taxol exposure	Antagonistic
	24 hour ET-743 followed by 24 hour exposure to SN38	Additive/Synergistic
	24 hour SN-38 followed by 24 hour exposure to ET-743	Additive/Synergistic
	24 hour concurrent ET-743/SN-38 exposure	Additive
NCB-N23	24 hour ET-743 followed by 24 hour exposure to taxol	Additive/Antagonistic
	24 hour taxol followed by 24 hour exposure to ET-734	Additive
	24 hour concurrent ET-743/taxol exposure	Antagonistic
	24 hour ET-743 followed by 24 hour exposure to SN38	Additive
	24 hour SN-38 followed by 24 hour exposure to ET-743	Synergistic
	24 hour concurrent ET-743/SN-38	Additive/Synergistic

	exposure	
<u>Breast</u>		
MDA-435	24 hour ET-743 followed by 24 hour exposure to gemcitabine	Additive
	24 hour gemcitabine followed by 24 hour exposure to ET-743	Additive
	24 hour concurrent ET-473/gemcitabine	Additive
MDA-231	24 hour ET-743 followed by 24 hour exposure to gemcitabine	Additive
	24 hour gemcitabine followed by 24 hour exposure to ET-743	Additive
	24 hour concurrent ET-473/gemcitabine	Additive
T47-8	24 hour ET-743 followed by 24 hour exposure to gemcitabine	Additive
	24 hour gemcitabine followed by 24 hour exposure to ET-743	Additive
	24 hour concurrent ET-473/gemcitabine	Additive
<u>Colon</u>		
MCT-116	24 hour ET-743 followed by 24 hour exposure to SN-38	Synergistic
	24 hour ET-743 followed by 24 hour exposure to SN-38	Additive
	24 hour concurrent ET-743/SN exposure	Additive
NT-29	24 hour ET-743 followed by 24 hour exposure to SN-38	Additive
	24 hour ET-743 followed by 24 hour exposure to SN-38	Additive
	24 hour concurrent ET-743/SN exposure	Additive
Colo-320	24 hour ET-743 followed by 24 hour exposure to SN-38	Additive
	24 hour ET-743 followed by 24 hour exposure to SN-38	Additive/Synergistic
	24 hour concurrent ET-743/SN exposure	Synergistic

<u>rhabdomyo-</u> <u>sarcoma</u>		
RN1	24 hour ET-743 followed by 24 hour exposure doxorubicin	Additive
	24 hour doxorubicin followed by 24 hour exposure to ET-743	Additive
	24 hour concurrent ET-743/doxorubicin exposure	Antagonistic
RD	24 hour ET-743 followed by 24 hour exposure doxorubicin	Additive
	24 hour doxorubicin followed by 24 hour exposure to ET-743	Additive
	24 hour concurrent ET-743/doxorubicin exposure	Additive/Antagonistic
RN30	24 hour ET-743 followed by 24 hour exposure doxorubicin	Additive
	24 hour doxorubicin followed by 24 hour exposure to ET-743	Additive
	24 hour concurrent ET-743/doxorubicin exposure	Antagonistic

Conclusions-Summary

These studies suggest that regardless of sequence of exposure between ET-743 and standard chemotherapy agents, an additive pattern of drug-drug interaction is most typically observed.

Evidence of synergy was observed when NC1-H522 and NC1-H23 NSCL lines were pre-exposed to SN-38, pre-exposure to ET-743 with cisplatin against HOS osteosarcoma, T-470 breast cell line with gemcitabine, SN-38 against HCT-116 colon, and concurrent exposure with SN-38 against Colo-320 colon tumor cell line.

Evidence of antagonism was observed when taxol was utilized concurrently against the NC1-H226 and NC1-H23 NSCL cell line and doxorubicin against the RHI rhabdomyosarcoma tumor cell line.

Example 5

Interaction Between Et-743 And Other Antineoplastic Agents

Although ET-743 is presently in clinical trials from human cancers, the mechanisms of antitumor activity of ET-743 have not been completely elucidated. The aim of this study was to assess the nature of the interaction between ET-743 and other antineoplastic agents (doxorubicin; DXR, trimetrexate; TMTX and Paclitaxel; Taxol) using the combination index (CI) method of Chou and Talalay. To better understand how ET-743 might be used clinically, the present study used SRB assays to examine the cytotoxicity resulting from combining ET-743 with three other antineoplastic agents in the different administration schedules in two soft tissue sarcoma cell lines, HT-1080 and HS-18, in vitro. DXR was the only agent that resulted in sequence-independent synergy when combined with ET-743. Concurrent exposure of ET-743 with DXR resulted in synergistic interactions in both cells lines.

The CIs (mean) with the schedule were 0.86, 0.83, 0.84 and 0.85 at 50, 75, 90 and 95% cell kill, respectively, in HT-1080 cells and 0.89, 0.74, 0.64 and 0.60 at 50, 75, 90 and 95% cell kill, respectively, in HS-18 cells. Sequencing with ET-743 for 24 h prior to DXR was the most effective regimen against both cell lines; it resulted in consistently low CI of up to the about 90% cell kill level for both cell lines. Exposure to Taxol prior to ET-743 was also an effective regimen. These results suggest that the combination of ET-743 and DXR should be explored further in clinical trials in the treatment of soft tissue sarcoma.

Materials And Methods

Chemicals

ET-743 was provided by Pharma-Mar S.A (Tres Cantos, Madrid, Spain), and was prepared as a 2 mM stock solution in dimethyl sulfoxide. Paclitaxel and DXR were obtained from Sigma chemical Co. (St. Louis, MO). TMTX was supplied by Warner-Lambert (Parke-Davis, Ann Arbor, Mich).

Cell Culture

Soft tissue sarcoma cell lines, HT-1080 and HS-18 were maintained as monolayer cultures in RP<I-1640 containing 10% fetal bovine serum.

SRB Cytotoxicity Assay

Cytotoxicity to drugs was determined by SRB cytotoxicity assay carried out in 96-well microtiter plates as described. Cells were plated in duplicate wells (5000 cells/well) and exposed to drugs at different concentrations. Cells were fixed with 50% TCA solution for 1 h and 0.4% SRB (Sigma) was added to each well. After a 30 min incubation, the plate were washed with 1% acetic acid and read at 570 nm on a Biowhitaker microplate reader 2001. The wells with cells containing no drugs and with medium plus drugs but no cells were used as positive and negative controls, respectively.

Concurrent Exposure to ET-743 and DXR, TMTX or Paclitaxel

Cells were seeded into 96-well plates, as described previously. Cells were treated with seven different concentrations of the single drugs or combinations mixture at 1:100 (ET-743 : the other drugs) molar ratio. After 72 h exposure, growth inhibition was measured using the SRB assay.

Sequential Exposure to ET-743 and DXR, TMTX or Paclitaxel

Using the same experimental setup described above, we exposed cells to three different concentrations of drugs which represents the IC₂₅, IC₅₀, IC₇₅ of ET-743, DXR, TMTX and paclitaxel, respectively. After 24 hours pre-treatment with ET743 or the combination

drug, the second drugs were added to the respective wells for 48 h. Growth inhibition was determined using the SRB assay.)

Cell Cycle Analysis

Exponentially growth cells were treated with or without drugs for several hours. Cells were then collected and fixed with ice-cold 70% methanol. DNA was stained with propidium iodide as described previously. Ten thousand stained cells were analyzed on a Becton Dickinson fluorescence-activated cell sorter (FACS).

Determination of Synergism and Antagonism and Construction of Isobolograms

The CI was calculated by the Chou-Talalay equation, which takes into account both potency (D_m or IC_{50}) and the shape of the dose effect curve (the m value). The general equation for the classic isobologram ($CI = 1$) is given by:

$$CI = (D)_1 / (Dx)_1 + (D)_2 / (Dx)_2 \quad (A)$$

where $(Dx)_1$ and $(Dx)_2$ in the denominators are the doses (or concentrations) for D_1 (ET-743) and D_2 (another drug) alone that give $X\%$ inhibition, whereas $(D)_1$ and $(D)_2$ in the numerators are doses of ET-743 and another drug in combination also inhibited $X\%$ (ie isoeffective). $CI < 1$, $CI = 1$, $CI > 1$ indicated synergism, additive effect and antagonism, respectively.

The $(Dx)_1$ or $(Dx)_2$ can be readily calculated from the median-effect equation of Chou and Chou et al:

$$Dx = D_m [fa / (1 - fa)]^{1/m} \quad (B)$$

where D_m is the median-effect dose that is obtained from the anti-log of the X-intercept of the median-effect plot, $X\text{-log}(D)$ versus $Y\text{-log}[fa / (1 - fa)]$ or

$D_m = 10^{-(Y\text{-intercept}) / m}$, and m is the slope of median-effect plot. Computer software of Chou and Chou allows automated calculation of m , D_m , Dx , and CI values. From $(Dm)_1$, $(Dx)_2$, and $D_1 + D_2$, it becomes easy to construct isobolograms automatically based on Eq. A.

For conservative mutually nonexclusive isobolograms of two agents, a third term,

$$(D1) (D2) / (DX)_1 (DX)_2$$

(C)

is added to Eq. A.

For simplicity, the third term is usually omitted, and thus the mutually exclusive assumption or classic isobologram is indicated. In Result 2 and 3, the CI values obtained from the classic (mutually exclusive) calculation are given.

Result 1

Cytotoxicity of four drugs on HT-1080 and S18			
IC ₅₀ for human soft tissue sarcoma cells			
		HT-1080	HS-18
ET-743	(nM)	0.01	0.27
DXR	(nM)	25	225
TMTX	(nM)	6	70000
Paclitaxel	(nM)	1.3	10

This table showed that both HT-1080 and S18 cell lines were more sensitive to ET-743 than other antineoplastic agents.

Effect of each agent on cell cycle distribution against HS-18 cells 24 h and 72 h after treatment with approximate IC ₅₀ dose					
Drugs	Dose	HR	%G1	%S Phase	%G2-M
Control			76.3	11.2	12.5
ET-743	270 pM	24	32.4	47.6	20.0
		72	86.7	8.4	4.9
DXR	225 nM	24	10.1	64.9	25.0
		72	1.3	63.8	34.9
TMTX	70 uM	24	44.2	53.8	1.9
		72	35.5	57.6	7.0
Paclitaxel	10 nM	24	32.8	52.5	15.5

		72	23.5	58.7	26.2
--	--	----	------	------	------

Effect of each agent on cell cycle distribution against HT-1080 cells 24 h and 72 h after treatment with approximate IC ₅₀ dose					
Drugs	Dose	Hr	%G1	%S phase	%G2-M
Control			47.5	35.8	16.7
ET-743	10 pM	24	42.6	36.1	21.3
		72	83.1	10.2	6.7
DXR	25 nM	24	36.1	17.5	46.4
		72	46.2	5.3	48.5
TMTX	6 nM	24	31.9	56.8	11.3
		72	32.0	53.7	14.4
Paclitaxel	1.3 nM	24	45.4	37.3	17.3
		72	86.0	9.0	5.0

Result 2 shows the CI for HT-1080 and HS-18 cells, respectively, which were simultaneously exposed to ET-743 and one of antineoplastic drugs, such as DXR, TMTX or paclitaxel, at 1 to 100 molar ratio combination mixture. When cells were treated with ET-743 and DXR, the CI values were all below 1, indicating synergism effect in both cell lines. The CI (mean) with this schedule were 0.86, 0.83, 0.84 and 0.85 at 50, 75, 90 and 95% cell kill, respectively, in HT-1080 cells and 0.89, 0.74, 0.64 and 0.60 at 50, 75, 90 and 95% cell kill, respectively, in HS-18 cells. This result showed that concurrent treatment of ET-743 and DXR produced synergistic cytotoxic effect. In contrast, when cells were treated with ET-743 and TMTX or paclitaxel, antagonism cytotoxic effect was observed.

The CI plot was obtained from both cell lines which were initially exposed to ET-743 for 24 h, followed by DXR for 48 h. In both cell lines, ET-743 followed by DXR treatment showed synergistic cytotoxic effect, the CI value of HT-1080 at 80% cell kill level was 0.64 ± 0.12 and that of HS-18 at 88% cell kill level was 0.24 ± 0.06 . In contrast, DXR followed by ET-743 treatment (Result 3a, lower figure) demonstrated the good CI value at first sight however, the CI value of HT-1080 at 80% cell kill level was 1.00 ± 0.03 , indicating that the

effect of the two agents were additive, in addition, the CI at highest fraction killed was worse than that at middle fraction killed in both cells.

When cells were exposed to ET-743 followed by TMTX, the CI values of HT-1080 showed nearly one or over one, indicating that the effect of the two agents are antagonism or additive. In contrast, those of HS-18 were all under 0.6, demonstrating that these two drugs have synergy effect. When cells were treated with TMTX followed by ET-743, additive effect was observed in both HT-1080 and HS-18 cell lines.

Paclitaxel followed by ET-743 treatment produced synergistic cytotoxic effect. When cells were exposed to paclitaxel followed by ET-743, the CI value of HT-1080 at 89% cell kill level was 0.92 ± 0.06 and that of HS-18 at 78% cell kill level was 0.38 ± 0.13 .

Summary

ET-743 was highly active against human soft tissue sarcoma cells, especially against the malignant fibrosarcoma cell line HT-1080.

DXR resulted in sequence-independent synergy when combined with ET-743, however, sequencing with ET-743 followed by DXR was more effective against both cell lines.

Exposure to paclitaxel followed by ET-743 was also an effective regimen against human soft tissue sarcoma cells, while concomitant exposure was antagonistic.

Example 6

In vivo combinations of chemotherapeutic agents with Ecteinascidin 743 (Et743) against solid tumors.

Several unique mechanisms of action have been described for Et743 including binding to the minor groove of DNA, alkylation of the N2 of guanine, transcriptional inhibition of MDR1 gene (Jin et. al., PNAS 97, 6775, 2000; Minuzzo et. al., PNAS 97, 6780, 2000) and

counteracting the activation of nuclear receptor SXR (Synold et. al., Nature Med 7, 584, 2001). As a single agent, Et743 inhibits *in vivo* tumor growth achieving complete remissions (CR) against several human tumor strains (Hendriks et. al., Ann Oncol 10, 1233, 1999) including melanoma (MEXF 989), NSCL (LXFL 529), ovary (HOC 22) and breast carcinoma (MX-1). The effectiveness of Et743 in combination with drugs that work by alternate mechanisms may provide opportunities to reduce the toxicities of either drug or to potentiate the effectiveness of a drug in resistant or relapsed cancers.

For this evaluation several agents including doxorubicin (DOX; 8 mg/Kg), cisplatin (DDP; 12 mg/Kg) and vinblastine (VINB; 6 mg/Kg) were administered before/after Et743 (0.2 mg/Kg) with 1 hour pretreatment, qdx5, in one or more of the following tumors: chondrosarcoma (CSHA), osteosarcoma (OSA-FH), fibrosarcoma (SW684), ovary (MRI-H-1834), NSCL (LX-1) and renal (MRI-H-121) with activity defined as < 50% T/C. In the hollow fiber (HF) model, the sequence of DOX, 1 hr pre-Et743 was consistently more effective than Et743 alone in chondrosarcoma (6% vs. 10%), fibrosarcoma (33% vs. 48%) and osteosarcoma (20% vs. 34%). Osteosarcoma xenografts produced similar results of 17% vs. 43%. HF studies with DPP showed that Et743 pre-DDP was more effective than Et743 alone in ovary (28% vs. 100%) and chondrosarcoma (15% vs. 19%) and equivalent activities in osteosarcoma (36% T/C). Xenograft data confirms the sequence of Et743 pre-DDP as more effective than Et743 alone (35% vs. 66%). The one exception was in NSCL where Et743 alone was not active (62% T/C) but DPP followed by Et743 produced CR (<1% T/C). In renal xenografts Et743 alone was very active (22% T/C) but Et743 followed by VINB also produced CR (<1% T/C). Separate studies are underway with other standard agents in breast, renal, melanoma and gastric tumor xenografts.

Example 7

Preclinical activity and biodistribution of Ecteinascidin 743 (ET-743) and Doxorubicin (DOX) combinations in human rhabdomyosarcoma.

ET-743 is the first of a new class of antitumor agents that exhibits anti-tumor activity. ET-743 has shown activity in patients with sarcoma refractory to DOX and ifosfamide. In

view of its potential as an effective drug, we investigated (1) the preclinical anti-tumor activity of ET-743/DOX combination against the human rhabdomyosarcoma TE 671 and (2) possible interactions between the drugs and their biodistribution in nude mice and tumor xenografts.

In vitro: The effect of each drug or combination after 1 hr exposures was evaluated by clonogenic assay. ET-743 or DOX alone showed anti-tumor activity against TE 671 cells. The combination according to isobologram analysis and Combination Index, was at least additive in several tumor cell lines including TE 671.

In vivo: Single iv treatments (ET-743, 0.1mg/Kg; DOX, 10mg/Kg) were administered in nude mice when xenograft tumors weighed approximately 100 mg. Tumor weight inhibition/Log10 Cell Kill values were 46%/0.132 for ET alone, 50%/0.33 for DOX alone, 77%/0.924 for ET-743 and DOX given simultaneously, 82%/1.12 for the combination of ET-743 given 1h before DOX, and 75%/0.85 when ET-743 was given 1h after DOX. A synergistic effect has also been observed against the murine fibrosarcoma UV2237 and against its multidrug resistant subline UV2237/ADR.

These data show a synergistic effect of ET-743/DOX and appears to be independent of drug sequence or combination in the scenarios studied thus far. Neither the plasma nor the tumor concentrations of DOX are significantly different when DOX was given alone or in combination with ET-743. The pharmacokinetic (PK) evaluation of ET-743 given alone or in combination with DOX is underway. The combination of ET-743 and DOX appears additive in vitro yet synergistic in vivo in rhabdomyosarcoma TE 671. The PK profile of DOX is not influenced by concomitant treatment with ET-743. These data provide a rationale for using this combination in early clinical trials.

Example 8

ET-743 and cisplatin (DDP) show in vitro and in vivo synergy against human sarcoma and ovarian carcinoma cell lines.

We show here that ET-743 enhances the activity of DDP both *in vitro* and *in vivo*. In several cancer cell lines including human intestinal carcinoma (HCT116), ovarian carcinoma (Igrov-1, A2780), their resistant sublines (Igrov-1/PSC-ET and 1A9, respectively), and rhabdomyosarcoma (TE671), lower concentrations of ET-743 used as a single agent could potentiate DDP activity by at least 2-fold. Concentrations corresponding to IC30/IC50 of ET-743 resulted in either additive or synergistic effects. These results have led to *in vivo* studies using xenograft models to study effective drug combinations with ET-743.

In *sc* transplanted TE671, partially sensitive to either ET-743 and DDP, the combination of the two drugs produced an antitumor effect much greater than that achieved with either drug used at their respective MTD levels. The ovarian 1 A 9 tumor that is normally resistant to both ET-743 and DDP as single agents, in combination produced a tumor growth inhibition greater than 50%. Orthotopically transplanted human ovarian carcinoma HOC8, producing tumor nodules in the peritoneal cavity with ascitis, which is resistant to ET-743 and partially sensitive to DDP, in combination resulted in a dramatic increase in survival even at the dose of ET-743 of 0.05 mg/Kg (1/4 MTD) and did not cause any significant toxicity. An ET-743 dose of 0.15 mg/Kg markedly increased survival, but there was also an increase in toxicity as indicated by a weight loss, that was significantly higher than that observed after treatment with each drug.

These findings offer a strong rationale to design clinical trials using the combination of ET-743 and DDP in sarcomas and ovarian cancers. *In vitro* and *in vivo* studies are in progress to elucidate the mechanisms underlining the synergism between ET-743 and DDP in these cancer types.

Example 9

High-Dose Dexamethasone (dex) Protects Against the Hepatotoxicity of Ecteinascidin-743 (ET-743) in the Rat

ET-743, an agent derived from a marine tunicate, is currently in phase II clinical trial. It has shown clinical activity against sarcomas, and preliminary data suggests activity against

breast and ovarian carcinoma. However, hepatotoxicity characterized by reversible transaminitis occurs in most treated patients and cholestasis in a minority. In the most sensitive animal species, the rat, toxicity of ET-743 is characterized by hepatic necrosis and bile duct inflammation. In the light of the antiinflammatory activity of dex, we investigated its effect on liver damage induced by ET-743 in the rat. Female Wistar rats received a single *iv* dose of ET-743 (40 µg/kg). Some rats were pretreated with a single oral dose of dex either at 1, 5, 10 or 20 mg/kg 24 h prior to ET-743 treatment. Liver pathology and plasma concentrations of alkaline phosphatase (ALP), aspartate aminotransferase (GOT) and total bilirubin (TB) were assessed up to 3 days *post* ET-743 administration. Conventional histological sections of the livers were examined by light microscopy.

At 2 days *post* ET-743 treatment, livers from rats that received ET-743 alone showed bile duct inflammation, striking degenerative changes in biliary epithelial cells and zones of hepatic necrosis. Plasma levels of ALP and GOT were significantly elevated after 2 days. Cholestasis was reflected by a dramatic increase in plasma TB concentrations, which commenced on day 2 after ET-743. ET-743-induced histopathological changes and elevation of plasma ALP, GOT and TB were totally abrogated in rats pre-treated with 10 or 20 mg/kg dex.

Whilst dex at 1 mg/kg showed little protection, 5 mg/kg was moderately protective. Plasma levels of ET-743 in rats which received dex (50 mg/kg) daily for 3 days prior to ET-743 were not decreased compared to those in rats on ET-743 alone. Furthermore, the activity of ET-743 against B16 melanoma implanted into mice was not impeded by dexamethasone. These findings suggest that the addition of high-dose dexamethasone to the ET-743 regimen may ameliorate its hepatotoxicity in cancer patients.

CLAIMS

1. The use of ET-743 in the preparation of a medicament for an effective treatment of a tumour by combination therapy employing ET-743 with another drug.
2. The use of a drug in the preparation of a medicament for an effective treatment of a tumour by combination therapy employing the drug with ET-743.
3. The use according to claim 1 or 2, where the combination of ET-743 and the drug is synergistic.
4. The use according to claim 1, 2 or 3, where the ET-743 forms part of the same medicament, or is provided as a separate medicament for administration at the same time or a different time as the drug.
5. The use according to any preceding claim, wherein the combination therapy employs ET-743 and an anthracycline
6. The use according to claim 5, wherein the combination therapy employs ET-743 and doxorubicin.
7. The use according to any of claims 1 to 4, wherein the combination therapy employs ET-743 and a platinum antitumour compound.

8. The use according to claim 7, wherein the combination therapy employs ET-743 and cisplatin.
9. The use according to any preceding claim, wherein the combination thereapy employs ET-743 and dexamethasone.
10. ET-743, and an anti-tumour drug synergistic with the ET-743 upon administration to a patient with a tumour.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
10 May 2002 (10.05.2002)

PCT

(10) International Publication Number
WO 02/036135 A3

(51) International Patent Classification⁷: **A61K 45/06**,
31/495, A61P 35/00

Institute de la Recherche Farmacologique Mario Negr, i, I-
Milan (IT). **GESCHER, Andreas** [DE/GB]; 7 Swithland
Court, Brand Hill, Woodhouse Eaves LE12 8SS (GB).

(21) International Application Number: PCT/GB01/04902

(74) Agent: **RUFFLES, Graham, Keith**; Marks & Clerk,
57-60 Lincoln's Inn Fields, London WC2A 3LS (GB).

(22) International Filing Date:
6 November 2001 (06.11.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/246,233 6 November 2000 (06.11.2000) US
60/248,095 13 November 2000 (13.11.2000) US
60/345,982 19 October 2001 (19.10.2001) US

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,
MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG,
SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN,
YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF,
CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD,
TG).

(71) Applicant (*for all designated States except US*):
PHARMA MAR, S.A. [ES/ES]; Calle de la Calera,
3, Poligono Industrial de Tres Cantos, Tres Cantos,
E-28760 Madrid (ES).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): **TAKAHASHI,**
Naoto [JP/US]; Memorial Sloan Kettering 1275 York
Avenue, New York, NY 10021 (US). **WEITMAN, Steve**
[US/US]; Institute for Drug Development, Cancer Ther-
apy and Research Center (CTRC), 14960 Omicron, San
Antonio, TX 78245-3217 (US). **D'INCALCI, Maurizio**
[IT/IT]; Institute de Rechercher Farmacologique Mario Ne-
gri, I- Milan (IT). **FAICLOTH, Glynn, Thomas** [US/US];
PharmaMar USA, Inc., 320 Putnam Avenue, Cambridge,
MA 02139-4616 (US). **GIAMAZZI, Rafaella** [IT/IT];

Published:

— with international search report

(88) Date of publication of the international search report:
10 April 2003

*For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.*

(54) Title: COMPOSITIONS FOR ANTITUMOUR TREATMENT CONTAINING ECETINASCIDIN 743

(57) Abstract: ET-743 is used in the preparation of a medicament for an effective treatment of a tumour by combination therapy employing ET-743 with another drug.



WO 02/036135 A3

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 01/04902

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K45/06 A61K31/495 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 256 663 A (SAKAI RYUICHI ET AL) 26 October 1993 (1993-10-26) -----	

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- * & * document member of the same patent family

Date of the actual completion of the international search

3 December 2002

Date of mailing of the international search report

09/12/2002

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
 Fax: (+31-70) 340-3016

Authorized officer

Stienon, P

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 01/04902

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5256663	A	26-10-1993	US 5089273 A 18-02-1992
			US 5149804 A 22-09-1992
			AT 69234 T 15-11-1991
			AU 589282 B2 05-10-1989
			AU 7581987 A 11-01-1988
			DE 3774435 D1 12-12-1991
			EP 0309477 A1 05-04-1989
			FI 885726 A 09-12-1988
			JP 2562162 B2 11-12-1996
			JP 1502749 T 21-09-1989
			WO 8707610 A2 17-12-1987
			AT 159022 T 15-10-1997
			AU 650829 B2 30-06-1994
			AU 9176491 A 25-06-1992
			DE 69127905 D1 13-11-1997
			DE 69127905 T2 28-05-1998
			DK 559838 T3 18-05-1998
			EP 0559838 A1 15-09-1993
			ES 2111631 T3 16-03-1998
			GR 3025837 T3 30-04-1998
			JP 6503579 T 21-04-1994
			JP 3098029 B2 10-10-2000
			WO 9209607 A1 11-06-1992
